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Tenacibaculum aestuarii sp. nov., isolated from a tidal flat sediment in Korea

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A novel *Tenacibaculum*-like bacterial strain, SMK-4^T, was isolated from a tidal flat sediment in Korea. Strain SMK-4^T was Gram-negative, pale yellow-pigmented and rod-shaped. It grew optimally at 30–37 °C and in the presence of 2–3 % (w/v) NaCl. It contained MK-6 as the predominant menaquinone and iso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH and $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH as the major fatty acids (>10 % of total fatty acids). The DNA G+C content was 33·6 mol%. Phylogenetic trees based on 16S rRNA gene sequences showed that strain SMK-4^T fell within the evolutionary radiation encompassed by the genus *Tenacibaculum*. Strain SMK-4^T exhibited 16S rRNA gene sequence similarity levels of 95·2–98·6 % with respect to the type strains of recognized *Tenacibaculum* species. DNA–DNA relatedness levels and differential phenotypic properties made it possible to categorize strain SMK-4^T as a species that is separate from previously described *Tenacibaculum* species. On the basis of phenotypic properties and phylogenetic and genetic distinctiveness, strain SMK-4^T (=KCTC 12569^T=JCM 13491^T) should be classified as a novel *Tenacibaculum* species, for which the name *Tenacibaculum aestuarii* sp. nov. is proposed.

The genus *Tenacibaculum* was first proposed as a result of the reclassification of two species that had been assigned to the genus *Flexibacter*, i.e. *Flexibacter maritimus* (Wakabayashi *et al.*, 1986) and *Flexibacter ovolyticus* (Hansen *et al.*, 1992). The genus *Tenacibaculum* accommodates bacteria that are Gram-negative, generally rod-shaped and yellow-pigmented and is characterized chemotaxonomically by having MK-6 as the predominant menaquinone and by DNA G+C contents of 30·0–35·2 mol% (Suzuki *et al.*, 2001; Frette *et al.*, 2004; Yoon *et al.*, 2005; Choi *et al.*, 2006). *Tenacibaculum* species have been isolated from various marine environments including tidal flats, a diseased red sea bream fingerling, halibut eggs, sponges and macroalgae (Wakabayashi *et al.*, 1986; Hansen *et al.*, 1992; Suzuki *et al.*, 2001; Frette *et al.*, 2004; Yoon *et al.*, 2005; Choi *et al.*, 2006). At present, the genus *Tenacibaculum* consists of seven species with validly published names: *Tenacibaculum maritimum*, *Tenacibaculum ovolyticum*, *Tenacibaculum mesophilum* and *Tenacibaculum amylolyticum* (Suzuki *et al.*, 2001), *Tenacibaculum skagerrakense* (Frette *et al.*, 2004), *Tenacibaculum lutimaris* (Yoon *et al.*, 2005) and *Tenacibaculum litoreum* (Choi *et al.*, 2006). In this study, we report on the detailed taxonomic characterization of a *Tenacibaculum*-like bacterial strain, SMK-4^T.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SMK-4^T is DQ314760.

Levels of DNA–DNA relatedness between strain SMK-4^T and the type strains of some phylogenetically related *Tenacibaculum* species are presented in a supplementary table available in IJSEM Online.

Tidal sediments collected from Saemankum, Pyunsan, Korea, were used as the source for the isolation of bacterial strains. Strain SMK-4^T was isolated by the dilution plating technique on marine agar 2216 (MA; Difco) at 30 °C. Growth at various temperatures from 4 to 45 °C was measured on MA, and tolerance to various NaCl concentrations (0·5 %, w/v, and 1·0–9·0 %, w/v, using increments of 1·0 %) was measured in marine broth 2216 (MB; Difco). Growth in the absence of NaCl was investigated in R2A agar (Difco) and trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was used. The optimal pH and the pH range for growth were determined in MB adjusted to various pH values (pH 4·5–9·0, using increments of 0·5 pH units). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on MA and on MA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Cell morphology and flagellation were examined by using light microscopy (Nikon E600) and transmission electron microscopy on cells grown on MA. The Gram reaction was determined by using the bioMérieux Gram-stain kit according to the manufacturer's instructions. Gliding motility was investigated as described by Bowman (2000). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). Catalase and oxidase activities and the hydrolysis of casein and starch were determined as described by Cowan & Steel (1965). The hydrolysis of hypoxanthine, tyrosine and xanthine was performed on MA with the substrate concentrations reported previously (Cowan & Steel, 1965). The hydrolysis of

aesculin, gelatin and urea and the reduction of nitrate were studied as described by Lanyi (1987), with the modification that artificial seawater (containing per litre of distilled water, 23·6 g NaCl, 0·64 g KCl, 4·53 g $MgCl_2.6H_2O$, 5·94 g $MgSO_4.7H_2O$ and 1·3 g $CaCl_2.2H_2O$; Bruns *et al.*, 2001) was used for the preparation of media. The hydrolysis of Tweens 20, 40, 60 and 80 was determined as described by Cowan & Steel (1965), with the modification that artificial seawater was used for the preparation of media. Acid production from carbohydrates was determined as described by Leifson (1963). The utilization of substrates as sole carbon and energy sources was tested in a basal medium containing

Table 1. Differential phenotypic characteristics of *Tenacibaculum* species

Species: 1, *T. aestuarii* sp. nov.; 2, *T. lutimaris*, data from Yoon *et al.* (2005); 3, *T. skagerrakense*, data from Frette *et al.* (2004); 4, *T. amylolyticum*, data from Suzuki *et al.* (2001); 5, *T. mesophilum*, data from Suzuki *et al.* (2001); 6, *T. ovolyticum*, data from Suzuki *et al.* (2001); 7, *T. maritimum*, data from Suzuki *et al.* (2001); 8, *T. litoreum*, data from Choi *et al.* (2006). Symbols: +, positive; −, negative; W, weakly positive; V, variable reaction; ND, not determined; *n*, number of strains. Data in parentheses are for the type strains. All species are Gram-negative, rod-shaped and positive for catalase and oxidase activities.

Characteristic	1 (*n*=1)	2 (*n*=4)	3 (*n*=2)	4 (*n*=1)	5 (*n*=4)	6 (*n*=3)	7 (*n*=2)	8 (*n*=1)
Origin	Tidal flat, Korea	Tidal flat, Korea	Pelagic, Denmark	Macroalgae, Japan	Sponge and macroalgae, Japan	Halibut egg, Norway	Diseased red sea bream fingerling, Japan	Tidal flat, Korea
Cell size (μm)	0·3 × 2·0–3·5	0·5 × 2–10	0·5 × 2–15	0·4 × 2–5	0·5 × 1·5–10	0·5 × 2–20	0·5 × 2–30	0·3–0·5 × 2–35
Colony morphology								
Shape	Irregular, spreading edge	Irregular, spreading edge	Circular, spreading edge	Circular, spreading edge	Irregular, spreading edge	Regular edge	Uneven edge	Irregular, spreading edge
Diameter at 5 days (mm)	5–10	10–20	5–20	23–27	30–60	ND	<5	5–10
Colour	Pale yellow	Pale yellow	Bright yellow	Yellow	Yellow	Pale yellow	Pale yellow	Pale yellow
Gliding motility	+	+	−	+	+	+	+	+
Growth at pH 5·0	−	+	−	−*	−*	−*	−*	−
Temperature range (°C)	9–41	10–39	10–40	20–35	15–40	4–25	15–34	5–40
Optimal temperature (°C)	30–37	30–37	25–37	27–30	28–35	ND	30	35–40
Nitrate reduction	−	V (−)	+	W	−	+	+†	+
Utilization of:								
Casamino acids	+	+	ND	+	+	V	+	+
Sucrose	−	−	+	−	−	−	−‡	−
D-Ribose	−	−	ND	−	−	−	−‡	−
DL-Aspartate	−	−	+	−	+	−	−	−
L-Proline	−	−	+	+	+	−	−	+
L-Glutamate	−	−	+	+	+	−	W (−‡)	−
Enzyme activities (API ZYM)								
Esterase lipase (C8)	+	W*	ND	ND	ND	ND	ND	+
Lipase (C14)	−	−*	ND	ND	ND	ND	ND	−
Trypsin	−	+*	ND	ND	ND	ND	ND	+
Hydrolysis of:								
Casein	+	+	+	+	+	+	+	ND
Starch	−	−	+	+	−	−	−	+
Gelatin	+	+	ND	+	+	+	−	+
DNA G+C content (mol%)	33·6	32·3–32·8	35·2	30·9	31·6–32·0	30·3–32·0	31·3–32·5	30·0

*Data from Choi *et al.* (2006).

†Data from Suzuki *et al.* (2001); the opposite result was reported by Gosink *et al.* (1998).

‡Data from Suzuki *et al.* (2001); the opposite result was reported by Wakabayashi *et al.* (1986).

0·2 g $NaNO_3$, 0·2 g NH_4Cl and 0·05 g yeast extract in 1000 ml artificial seawater (Bruns *et al.*, 2001), as described by Suzuki *et al.* (2001). The API ZYM system (bioMérieux) was used to determine enzyme activities. Antibiotic sensitivities were tested by spreading a bacterial suspension on MA and applying discs impregnated with the following antibiotics (concentration per disc): ampicillin (10 μg), carbenicillin (100 μg), cephalothin (30 μg), gentamicin (30 μg), lincomycin (15 μg), kanamycin (30 μg), neomycin (30 μg), novobiocin (5 μg), oleandomycin (15 μg), penicillin G (20 U), polymyxin B (100 U), streptomycin (50 μg) and tetracycline (30 μg).

Strain SMK-4^T was cultivated for 2–3 days in MB at 30 °C to obtain the cell biomass required for isoprenoid quinone analysis and DNA extraction. Isoprenoid quinones were analysed as described previously (Komagata & Suzuki, 1987), using reverse-phase HPLC. For fatty acid methyl ester analysis, cell mass of strain SMK-4^T was harvested from MA plates after cultivation for 3 days at 30 °C. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Chromosomal DNA was extracted and purified by using the procedure described by Yoon *et al.* (1996). The DNA G+C content was determined by using the method of Tamaoka & Komagata (1984), with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reverse-phase HPLC.

The 16S rRNA gene amplification was performed according to the method described previously, using two universal primers (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene was performed as described by Yoon *et al.* (2003). Alignment of sequences was carried out with the CLUSTAL W program (Thompson *et al.*, 1994) and gaps at the 5′ and 3′ ends of the alignment were omitted from further analysis. The evolutionary distances were calculated, using the Kimura two-parameter correction, with the CLUSTAL W package (Thompson *et al.*, 1994). A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) on the basis of distance matrix data. The reliability of grouping was assessed by means of 1000 bootstrap resamplings of the neighbour-joining dataset obtained using the CLUSTAL W package. DNA–DNA hybridization was determined by using the microplate hybridization method (Ezaki *et al.*, 1989) with photobiotin-labelled DNA probes and microdilution wells. The type strains of four *Tenacibaculum* species were used as reference strains for DNA–DNA hybridization: *T. litoreum* KCCM 42115^T, which was obtained from the Korean Culture Center of Microorganisms (KCCM), Seoul, Korea; and *T. mesophilum* DSM 13764^T, *T. skagerrakense* DSM 14836^T and *T. lutimaris* TF-26^T, which were obtained in the study of Yoon *et al.* (2005).

The morphological, cultural, physiological and biochemical characteristics of strain SMK-4^T are shown in Table 1 or are given in the species description (see later). Strain SMK-4^T contained MK-6 as the predominant menaquinone (at a peak area ratio of approximately 91 %). The cellular fatty acid profile of strain SMK-4^T is shown in Table 2, together with those of several *Tenacibaculum* species. The fatty acid

Table 2. Cellular fatty acid content (%) of *Tenacibaculum aestuarii* sp. nov. and some other *Tenacibaculum* species

Strain: 1, *T. aestuarii* SMK-4^T; 2, *T. lutimaris* TF-26^T, data from Yoon *et al.* (2005); 3, *T. skagerrakense* DSM 14836^T, data from Yoon *et al.* (2005); 4, *T. mesophilum* DSM 13764^T, data from Yoon *et al.* (2005); 5, *T. maritimum* JCM 8137^T, data from Yoon *et al.* (2005); 6, *T. litoreum* KCCM 42115^T, data from Choi *et al.* (2006). Fatty acids that represented less than 0·5 % in all strains are omitted.

Fatty acid	1	2	3	4	5	6
Straight-chain fatty acids						
$C_{15:0}$	6·1	8·9	4·9	3·6	2·9	2·7
$C_{16:0}$	0·4	0·6	0·6	0·7	0·3	0·9
$C_{18:0}$	–	–	–	–	1·4	0·3
Branched fatty acids						
iso-$C_{13:0}$	1·3	0·7	0·2	0·8	1·8	1·4
iso-$C_{14:0}$	2·2	1·7	0·9	0·8	0·8	0·7
iso-$C_{15:0}$	18·9	17·2	9·5	13·2	16·8	18·8
iso-$C_{15:1}$	8·7	5·3	8·2	7·1	7·6	8·2
iso-$C_{16:0}$	2·0	3·8	1·3	1·7	0·3	2·3
iso-$C_{16:1}$	2·3	1·7	1·7	0·8	–	1·3
iso-$C_{17:1}\omega 9c$	0·8	0·4	–	0·6	–	1·6
anteiso-$C_{15:0}$	1·3	0·7	–	1·1	0·8	1·8
Unsaturated fatty acids						
$C_{15:1}\omega 6c$	3·0	4·2	–	1·6	2·2	1·7
$C_{17:1}\omega 6c$	1·6	1·5	1·2	0·9	0·3	0·9
$C_{18:3}\omega 6c$ (6,9,12)	–	–	–	–	–	1·5
Hydroxy fatty acids						
$C_{10:0}$ 3-OH	0·7	–	–	–	–	–
$C_{15:0}$ 2-OH	0·8	1·2	2·5	1·1	1·1	0·7
$C_{15:0}$ 3-OH	4·2	3·4	8·6	2·9	3·8	–
$C_{16:0}$ 3-OH	1·0	1·3	2·1	3·2	1·5	1·6
$C_{17:0}$ 2-OH	0·4	0·2	–	0·8	–	0·9
$C_{17:0}$ 3-OH	0·6	0·9	2·5	0·7	0·6	0·3
iso-$C_{14:0}$ 3-OH	0·5	–	–	–	–	–
iso-$C_{15:0}$ 3-OH	6·1	4·6	7·8	8·0	19·8	6·6
iso-$C_{16:0}$ 3-OH	12·3	12·8	12·2	9·0	5·0	6·8
iso-$C_{17:0}$ 3-OH	9·6	8·4	11·7	14·9	13·7	13·6
Unknown fatty acid (ECL* 13·565)	–	–	–	–	–	1·3
Unknown fatty acid (ECL* 16·582)	1·0	0·7	0·6	1·0	1·0	1·3
Summed feature†						
3	11·9	18·1	22·5	24·4	17·9	19·6
4	–	–	–	–	–	1·3

*ECL, equivalent chain-length.

†Summed feature represents groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained one or more of $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH. Summed feature 4 contained one or more of iso I-/anteiso-B-$C_{17:1}$.

profile was characterized by the presence of large amounts of branched, hydroxy, straight-chain and unsaturated fatty acids; the major components (>10 % of total fatty acids) were iso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH and $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (Table 2). The DNA G+C content of strain SMK-4^T was 33·6 mol%.

The 16S rRNA gene sequence of strain SMK-4^T determined in this study comprised 1473 nt. In the phylogenetic tree based on the neighbour-joining algorithm, strain SMK-4^T fell within the radiation of the cluster comprising *Tenacibaculum* species (Fig. 1). Strain SMK-4^T exhibited 16S rRNA gene sequence similarity levels of 97·3–98·6 % with respect to the type strains of *T. lutimaris*, *T. skagerrakense*, *T. mesophilum* and *T. litoreum*, and levels of 95·2–96·9 % with respect to the type strains of the other *Tenacibaculum* species. Sequence similarities with respect to other species included in the phylogenetic analysis were less than 93·4 %. The levels of DNA–DNA relatedness between strain SMK-4^T and the type strains of four *Tenacibaculum* species that showed 16S rRNA gene sequence similarity values of >97·0 % with respect to strain SMK-4^T were in the range 4·9–23·8 % (DNA–DNA relatedness levels are shown in Supplementary Table S1 in IJSEM Online).

The results obtained from chemotaxonomic analyses were consistent with the phylogenetic affiliation of strain SMK-4^T to the genus *Tenacibaculum*. The menaquinone type of strain SMK-4^T was the same as those of other *Tenacibaculum* species (Suzuki *et al.*, 2001; Yoon *et al.*, 2005; Choi *et al.*, 2006). The fatty acid profile of strain SMK-4^T was similar to those of *Tenacibaculum* species, although there were differences in the proportions of some fatty acids, perhaps as a result of different cultivation methods or analytical conditions (Table 2). The phenotypic characteristics of strain SMK-4^T could be distinguished from those of previously described *Tenacibaculum* species (Table 1). The phylogenetic distinctiveness and DNA–DNA relatedness data provide conclusive evidence that strain SMK-4^T differs from recognized *Tenacibaculum* species. On the basis of the data presented, strain SMK-4^T should be classified as a novel species of the genus *Tenacibaculum*, for which the name *Tenacibaculum aestuarii* sp. nov. is proposed.

Description of *Tenacibaculum aestuarii* sp. nov.

Tenacibaculum aestuarii (aes.tu′a.ri.i. L. gen. n. *aestuarii* of the tidal flat, from where the organism was isolated).

Cells are Gram-negative, rod-shaped, unflagellated and 0·3 × 2·0–3·5 μm. Colonies are smooth, pale yellow, irregular with spreading edges and greenish glistening. No growth occurs under anaerobic conditions on MA or on MA with nitrate. The optimal pH for growth is 7·5–8·5; weak growth occurs at pH 5·5, but there is no growth at pH 5·0. No growth occurs in the presence of more than 7 % (w/v) NaCl. Growth does not occur in the absence of NaCl. Flexirubin-type pigments are absent. Tyrosine and Tweens 20, 40, 60 and 80 are hydrolysed, but aesculin, urea, hypoxanthine and xanthine are not. Peptone and tryptone are utilized as sole carbon and energy sources. D-Glucose, D-galactose, D-fructose, D-cellobiose, D-trehalose and L-leucine are not utilized. Acid is not produced from D-sorbitol, *myo*-inositol, D-xylose, D-ribose, D-fructose, D-mannitol, melibiose, L-arabinose, D-melezitose, D-glucose, D-galactose, L-rhamnose, D-mannose, D-cellobiose, lactose, sucrose, maltose, D-trehalose or D-raffinose. Using the API ZYM system (bioMérieux), alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase, phosphohydrolase and β-glucosidase are present,

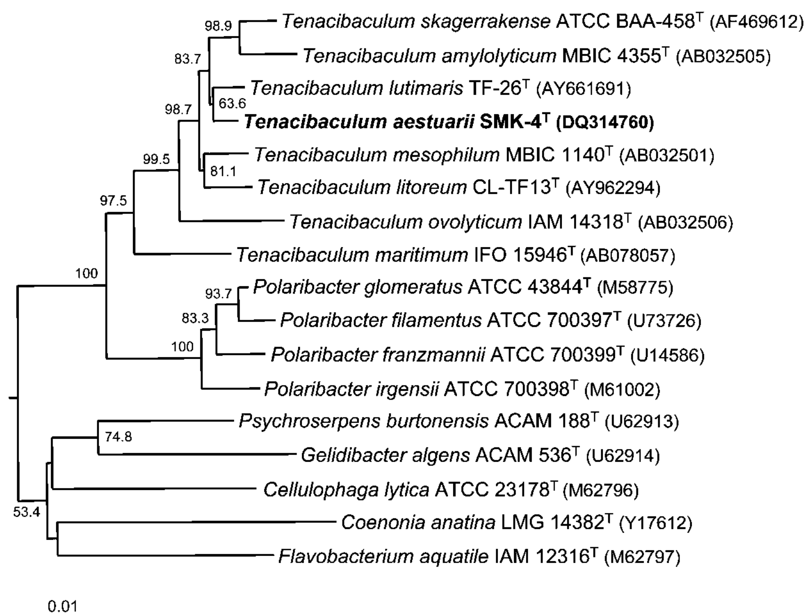

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain SMK-4^T and other related taxa, based on 16S rRNA gene sequences. Only those bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at the branching points. *Cytophaga hutchinsonii* (accession no. M58768) was used as an outgroup (not shown). Bar, 0·01 substitutions per nucleotide position.

but cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. Susceptible to cephalothin, lincomycin, oleandomycin and carbenicillin, but not to polymyxin B, streptomycin, penicillin G, ampicillin, gentamicin, novobiocin, tetracycline, kanamycin or neomycin. The major cellular fatty acids (> 10 % of total fatty acids) are iso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH and $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH. The predominant menaquinone is MK-6. The DNA G + C content is 33·6 mol%.

The type strain, SMK-4^{T} (= KCTC 12569^{T} = JCM 13491^{T}), was isolated from tidal flat sediment at Saemankum, Pyunsan, Korea.

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